Bacterial competition between a bacteriocin-producing and a bacteriocin-negative strain of *Streptococcus bovis* in batch and continuous culture

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Received 16 February 2006; revised 19 April 2006; accepted 23 April 2006.
First published online 12 June 2006.

DOI:10.1111/j.1574-6941.2006.00160.x

Editor: Gary King

Keywords
Streptococcus bovis; bacteriocins; bovicin HC5; bacterial competition.

Abstract

A bacteriocin-producing *Streptococcus bovis* strain (HC5) outcompeted a sensitive strain (JB1) before it reached stationary phase (pH 6.4), even though it grew 10% slower and cell-free bovicin HC5 could not yet be detected. The success of bacteriocin-negative *S. bovis* isolates was enhanced by the presence of another sensitive bacterium (*Clostridium sticklandii* SR). PCR based on repetitive DNA sequences indicated that *S. bovis* HC5 was not simply transferring bacteriocin genes to *S. bovis* JB1. When the two *S. bovis* strains were coinoculated into minimal medium, bacteriocin-negative isolates predominated, and this effect could be explained by the longer lag time (0.5 vs. 1.5 h) of *S. bovis* HC5. If the glucose concentration of the minimal medium was increased from 2 to 7 mg mL^{-1}, the effect of lag time was diminished and bacteriocin-producing isolates once again dominated the coculture. When the competition was examined in continuous culture, it became apparent that batch culture inocula were never able to displace a strain that had already reached steady state, even if the inoculum was large. This result indicated that bacterial selection for substrate affinity was even more important than bacteriocin production.

Introduction

Streptococcus bovis is not normally a predominant ruminal bacterium, but this opportunist can outgrow others if the diet has an abundance of soluble carbohydrates (Hungate *et al*., 1952). The success of *S. bovis* can be explained by its very fast growth rate. *Streptococcus bovis* grows twice as fast as any other ruminal bacterium, and it is able to generate more ATP per unit of time (Hungate, 1979). When it grows rapidly, *S. bovis* is homolactic, and ruminal lactic acid accumulation can be highly detrimental to the animal (Hungate *et al*., 1952; Nagaraja & Chengappa, 1998; Owens *et al*., 1998). Some strains of *S. bovis* produce bacteriocins (Jarvis, 1967), and *c*. 50% of the isolates from cattle fed hay or a diet based on grain produced bacteriocin-like substances (Mantovani *et al*., 2001). *Streptococcus bovis* HC5 produces only one bacteriocin (bovicin HC5), but it has a broad spectrum and it inhibits many other Gram-positive species (Mantovani *et al*., 2002).

Preliminary experiments indicated that bacteriocin-producing *S. bovis* HC5 grew more slowly than *S. bovis* JB1, a bacteriocin-negative strain, and previous work indicated that *S. bovis* HC5 did not secrete significant cell-free bovicin HC5 until it reached stationary phase (Mantovani & Russell, 2003a). The potential of *S. bovis* HC5 to inhibit other bacteria was also compromised by at least two additional observations. Bovicin HC5 is most active at pH values well below the normal ruminal range (Houlihan *et al*., 2004), and there was little binding of semi-purified bovicin HC5 to *S. bovis* JB1 until the pH was less than 6.0 (Houlihan & Russell, 2006). Given these constraints, the ability of bovicin HC5 to give *S. bovis* HC5 a competition advantage was not certain. The following experiments sought to examine the ability of a bacteriocin-producing strain of *S. bovis* (HC5) to compete with a sensitive strain (JB1). Competition studies were performed in continuous as well as in batch culture so the outcome could be more fully evaluated.

Materials and methods

Bacteria, media and growth

Streptococcus bovis JB1 and HC5 (Mantovani *et al*., 2001) were routinely grown under O_2-free CO_2 at 39 °C in basal

medium containing (L^{-1}): 292 mg K_2HPO_4 292 mg $KHPO_4$, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 600 mg cysteine hydrochloride, 1 g Trypticase (BBL Microbiology Systems, Cockeysville, MD), and 0.5 g yeast extract. The medium was adjusted to pH 6.7 with NaOH and autoclaved for 20 min. After the sterile medium had cooled to room temperature, sterile Na_2CO_3 (4 g L^{-1}) was added as a buffer. The minimal medium was the same as the basal medium except that the Trypticase and yeast extract were replaced by microminerals and vitamins (Cotta & Russell, 1982).

Cultures were grown in 18 × 150 mm tubes that were sealed with butyl rubber stoppers. Glucose was added to the basal medium after it had been autoclaved. Growth was monitored via changes in optical density (1 cm cuvette, 600 nm, Gilford 260 spectrophotometer). Growth rate was estimated from differences in the natural logarithm of optical density and time. *Clostridium sticklandii* was grown in a similar fashion, except that additional Trypticase (10 mg mL^{-1}) was substituted for glucose.

Competition studies

Streptococcus bovis JB1 and HC5 were inoculated (5% v/v) into basal medium that contained glucose (2 or 7 mg mL^{-1}). After 24 h of incubation (39 °C), the cultures were taken into an anaerobic glove box (Coy Laboratory Systems, Ann Arbor, MI) and diluted anaerobically in basal medium lacking glucose (10^5-, 10^6-, 10^7-fold). The aliquots (100 μL) from each dilution were spread on the surface of basal medium agar (2% w/v) that had been supplemented with glucose (10 mg mL^{-1}). The plates were incubated anaerobically (39 °C, 24 h). Twenty-five colonies were transferred from the agar plates to basal medium tubes supplemented with glucose (4 mg mL^{-1}) and incubated (39 °C, 24 h). Each isolate was examined for bacteriocin activity.

Bacteriocin activity

Streptococcus bovis JB1 was used as an indicator organism for *S. bovis* HC5 activity. Stationary phase *S. bovis* JB1 cultures (4 g glucose mL^{-1}) were inoculated into molten basal medium agar (0.1 mL into 100 mL) supplemented with 10 mg glucose mL^{-1}, and the agar was poured into Petri plates in an anaerobic glove box (Coy Laboratory Systems, Ann Arbor, MI). Once solidified, wells (5 mm diameter) were made in the agar. Cell-free supernatants from *S. bovis* cultures were diluted in twofold increments into basal medium lacking glucose, yeast extract and Trypticase. The diluted cell-free supernatants were then added to the agar wells. The plates were incubated at 39 °C for 24 h and examined for zones of clearing around the well. Activity units (AU mL^{-1}) were calculated from volume of the cell-free supernatant (100 μL) and the reciprocal of the highest serial dilution showing a visible zone of clearing.

Genetic analyses

Stationary phase *S. bovis* cultures were harvested by centrifugation (10 000 ***g***, 5 min, 22 °C) and washed once with TE buffer (10 mm Tris-Cl, 1 mm EDTA, pH 7.5). The cells were resuspended in distilled water (250 μg cell protein mL^{-1}) and mechanically disrupted (single-channel Mini-Bead Beater, Biospec Products, Bartlesville, OK; 50 s, highest setting) using sterile glass beads (100 μm diameter, Biospec Products). Cell debris was removed by centrifugation (7000 ***g***, 2 min, 22 °C), and the supernatant was diluted 10-fold with sterile water. The diluted extracts were used as templates for PCR analyses. Previous work indicated that *S. bovis* has repetitive DNA sequences interspersed throughout its genome, and these 'BOX' sequences have been used to differentiate strains as previously described (Jarvis *et al.*, 2001). The BOX-PCR was undertaken using the BOX1A primer (5′-ACACGGCAAGGCGACGCTGACG-3′), Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) using a PTC-200 Thermal Cycler (MJ Research, Incline Village, NV). The operational parameters were; 94 °C/3 min, followed by 30 cycles of 94 °C/45 s, 50 °C/1 min, 65 °C/6 min; and 65 °C/6 min. The PCR products were then separated with a 1% agarose gel (60 V, 8.6 V cm^{-1}, 60 min). The gel was stained with ethidium bromide and photographed using an Electrophoresis Documentation and Analysis System 120 (Eastman-Kodak Ltd, Rochester, NY).

Experimental design

Each competition experiment was performed three times and variation is shown by the standard deviation of the mean.

Results

Growth characteristics of *Streptococcus bovis JB1 and HC5*

Streptococcus bovis JB1 and HC5 both grew rapidly in basal medium (Fig. 1), but the maximum specific growth rate and final optical density of JB1 was always at least 10% greater. Because the glucose concentration was only 2 mg mL^{-1}, the final pH was 6.4. *Streptococcus bovis* JB1 and HC5 could also grow in a minimal medium that lacked yeast extract and Trypticase. Both *S. bovis* strains lagged in minimal medium, but the lag time of *S. bovis* HC5 was greater than JB1. After the lag phase, the growth rate of *S. bovis* JB1 and HC5 was approximately the same. When the glucose concentration was increased from 2 to 7 mg mL^{-1}, the final pH declined

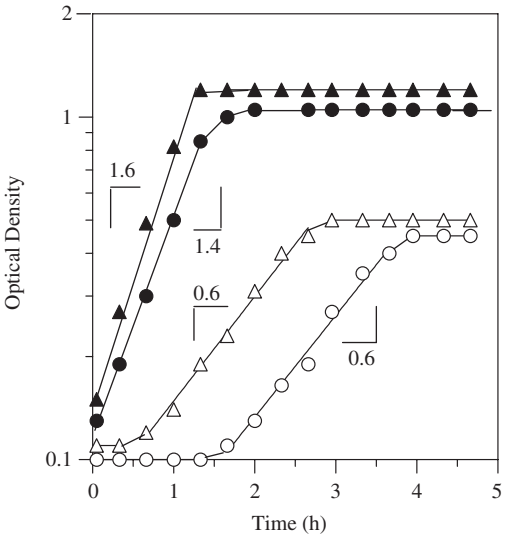

Fig. 1. The growth (optical density) of *Streptococcus bovis* HC5 (closed circles) and JB1 (closed triangles) in basal medium at pH 6.7. The growth (optical density) of *S. bovis* HC5 (open circles) and JB1 (open triangles) in minimal medium at pH 6.7. In each case, initial glucose concentration was 2 mg mL^{-1} and the final pH was 6.4. The maximum specific growth rates of each culture are also shown.

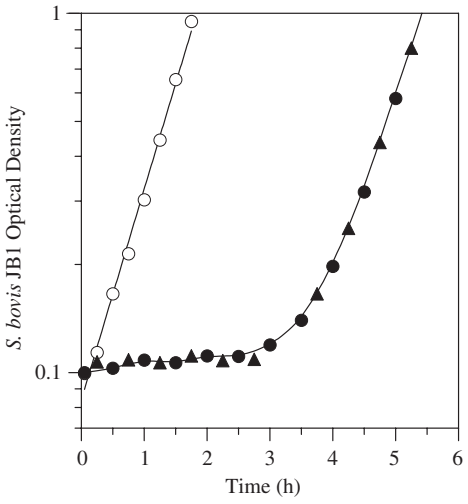

Fig. 2. The effect of cell-free *Streptococcus bovis* HC5 culture supernatant on the ability of stationary phase stationary *Streptococcus bovis* JB1 cells to reinitiate growth. The *S. bovis* JB1 cells were incubated (39 °C) in sterile filtered *S. bovis* HC5 culture supernatant (2 mg glucose mL^{-1}) for 0 h (open circles), 1 h (filled circles) or 2 h (filled triangles). The *S. bovis* JB1 cells were then inoculated into basal medium (2 mg glucose mL^{-1}) and incubated at 39 °C.

from 6.4 to 5.5. Stationary phase cultures always had at least 4 mm residual ammonia.

Effect of cell-free bovicin HC5 on *Streptococcus bovis* JB1

Cell-free supernatants from exponentially growing cells had no activity, but stationary phase *S. bovis* HC5 cultures had 320 and 1280 AU per mL when the glucose concentrations were 2 and 7 mg mL^{-1}, respectively. A *S. bovis* JB1 (5% inoculum) could not grow in sterile filtered cell-free supernatant that had been obtained from a stationary phase *S. bovis* HC5 culture (2 mg glucose mL^{-1}) even if supplemental glucose was added. However, small amounts (5%, v/v) of this same cell-free supernatant did not inhibit the growth of *S. bovis* JB1 in basal medium if the inoculum was 5% (v/v) (data not shown).

When stationary phase *S. bovis* JB1 cells (200 mg cell protein mL^{-1}) were harvested by centrifugation and resuspended in the sterile filtered *S. bovis* HC5 culture supernatant for as long as 24 h (39 °C), the viability of *S. bovis* JB1 did not decrease (data not shown). *Streptococcus bovis* JB1 cells that were harvested and suspended in sterile filtered *S. bovis* HC5 culture supernatant (1 h, 39 °C) lagged for *c.* 3 h after they were inoculated into basal medium (Fig. 2). If the exposure time was increased from 1 to 2 h, the lag time did not increase. These results indicated that the effect of the cell-free bovicin HC5 on *S. bovis* JB1 was bacteriostatic rather than bactericidal. Since no lag was observed if similar experiments were performed using cell-free supernatant from *S. bovis* JB1, the effect could not be explained by a decrease in culture pH or lactic acid accumulation (data not shown).

Competition between *Streptococcus bovis* JB1 and HC5 in basal medium

When *S. bovis* JB1 and HC5 were coinoculated (5%, v/v) into basal medium (2 mg glucose mL^{-1}) the percentage of bacteriocin-producing isolates initially decreased, but once the culture reached mid-log, most of the isolates produced bacteriocin (Fig. 3). By the time the coculture had reached late stationary phase (24 h) more than 90% of the isolates were bacteriocin-producers. If the late stationary phase cultures were transferred successively a second and third time, virtually all of the isolates were bacteriocin-producers. Similar results were obtained if the competition was performed in basal medium that had 3.5 times as much glucose (data not shown). The same results were obtained if a smaller inoculum was used (1%, v/v) and the outcome of the competition could only be counteracted by utilizing three times as much *S. bovis* JB1 as HC5 (data not shown).

Effect of *Clostridium sticklandii* SR on the *Streptococcus bovis* competition in basal medium

Because *C. sticklandii* SR does not utilize glucose and requires Trypticase as an energy source, it was possible to

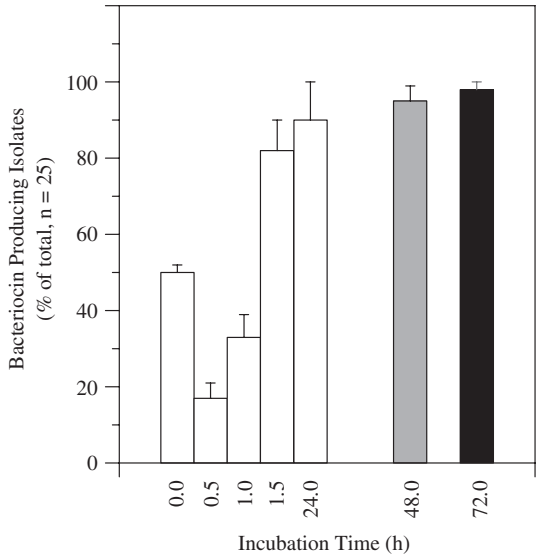

Fig. 3. Ability of bacteriocin-negative *Streptococcus bovis* isolates to compete with bacteriocin-producing *S. bovis* isolates at various incubation times in basal medium when the glucose concentration was 2 mg mL^{-1} and the final pH was pH 6.4. Open bars show the first transfer, gray bars the second transfer, and black bars the third transfer.

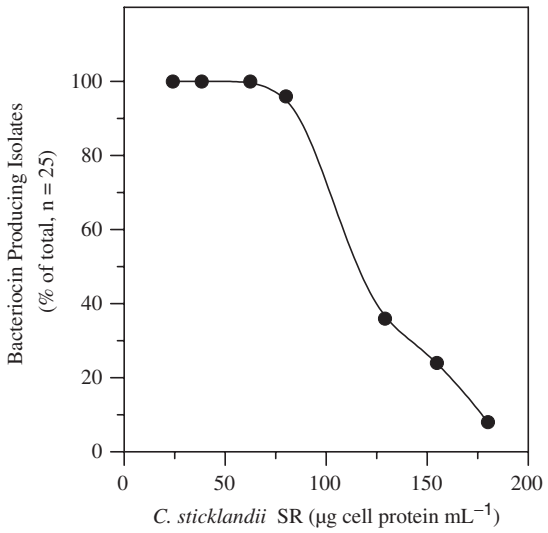

Fig. 4. The effect of *Clostridium sticklandii* SR on the bacteriocin-producing *Streptococcus bovis* isolates to compete with bacteriocin-negative *S. bovis* isolates. The experiment was conducted in two steps. *Clostridium sticklandii* SR was inoculated first and incubated for 16 h at 39 °C. The Trypticase concentration was varied from 1 to 10 mg mL^{-1} to alter the amount of *C. sticklandii* SR cell protein. Once *C. sticklandii* SR had reached stationary phase, glucose was added (2 mg mL^{-1}), and the incubation tubes were reinoculated with *S. bovis* HC5 and JB1 (5%, v/v). The tubes were then incubated for another 16 h at 39 °C. Isolates ($n = 25$) were tested for bovicin HC5 activity.

assess the impact of this sensitive bacterium on the competition between *S. bovis* strains by conducting a two-stage incubation. *Clostridium sticklandii* SR was inoculated into basal medium supplemented with various amounts of Trypticase. On the second day, the optical density and cell protein of *C. sticklandii* SR were determined. The *C. sticklandii* SR cells were harvested and resuspended in basal medium (2 mg mL^{-1} glucose), and *S. bovis* JB1 and HC5 were then coinoculated (5%, v/v). Results indicated that *C. sticklandii* SR cells promoted the success of bacteriocin-negative isolates, but only if the *C. sticklandii* SR cell protein concentration was greater than 75 μg mL^{-1} (Fig. 4). Because the number of colonies on basal medium plates containing glucose did not decrease even if large amounts of *C. sticklandii* SR cell protein were present, it appeared that the total *S. bovis* number was not being affected.

Competition between *Streptococcus bovis* JB1 and HC5 in minimal medium

When *S. bovis* JB1 and HC5 were coinoculated (5%, v/v) into minimal medium that contained 2 mg glucose mL^{-1}, bacteriocin-producing isolates were no longer the dominant type (< 25%). this outcome could be explained by the greater lag time of *S. bovis* HC5 when amino nitrogen and yeast extract were not available (data not shown). The success of bacteriocin-producing isolates in minimal medium was glucose-dependent. If the glucose concentration was increased from 2 to 7 mg glucose mL^{-1}, the impact of lag time was less, and more than 90% of the isolates produced bacteriocin (data not shown).

Competition in continuous culture (basal medium)

When *S. bovis* HC5 had reached steady state in a glucose-limited continuous culture (0.1 h^{-1} dilution rate, > 98% turnover of medium), the activity of the cell-free supernatant was nearly as great as activity observed with late stationary batch cultures (160 vs. 320 AU mL^{-1}). Nonetheless, even large inoculations of *S. bovis* JB1 (as great as 15%, v/v) did not alter the percentage of bacteriocin-producing isolates (100%, or 25/25 still positive) once a new steady state had been reached. However, the reverse was also true. If *S. bovis* JB1 had reached steady state in glucose-limited continuous culture, even repetitive (three times) very large inoculations of *S. bovis* HC5 (as large as 60%, v/v) did not alter the percentage of bacteriocin-producing isolates (0% or all negative), and cell-free bacteriocin activity from the culture vessel could not be detected. When *S. bovis* JB1 and HC5 were coinoculated (5%, v/v) into the culture vessel, the coculture grew rapidly before a steady state was reached. Once the steady state had been attained, none of the isolates produced bacteriocin.

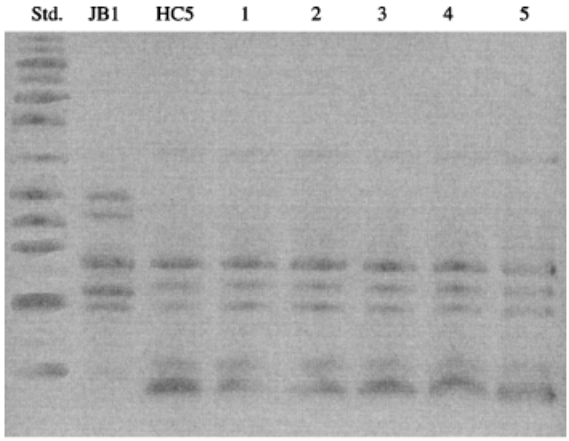

Fig. 5. An agarose gel showing the BOX-PCR patterns for *Streptococcus bovis* HC5 and JB1. The lane labeled Std shows a DNA ladder (> 500 bp).

Genetic transfer?

Streptococcus bovis HC5 and JB1 can be differentiated by their ability to produce bacteriocin, but this phenotype did not exclude the possibility that bacteriocin genes were being transferred from *S. bovis* HC5 to JB1 in the cocultures. *Streptococcus bovis* HC5 and JB1 have different BOX PCR patterns (Fig. 5), and this method allowed us to ascertain whether *S. bovis* JB1 had acquired an ability to produce bovicin HC5 from *S. bovis* HC5. All of the bacteriocin-producing isolates from batch cocultures had the same BOX PCR pattern as the HC5 strain.

Discussion

The idea that bacteria can produce antibacterial peptides is not new, but the impact of this antagonism on bacterial competition is not well understood. Eijsink *et al.* (2002) concluded that bacteriocin-producing bacteria would 'switch on bacteriocin production at times when competition for nutrients is likely to become more severe.' Tait & Sutherland (2002) indicated that the effect of bacteriocins could be even more complicated in natural environments covered by biofilms, but they speculated that bacteriocins would give 'a strain a competitive advantage when interacting with a bacteriocin-sensitive strain within a biofilm. The effect of bacteriocins on bacterial competition has typically been assessed by employing different species rather than strains within the same species' (Tait & Sutherland, 2002; Kreth *et al.*, 2005).

Our results indicated that *S. bovis* HC5 could outcompete *S. bovis* JB1, even though (i) *S. bovis* HC5 grew more slowly than *S. bovis* JB1; (ii) glucose concentration and maximal cell density of *S. bovis* HC5 was often low; (iii) the culture pH was suboptimal for bovicin HC5 activity and binding (Houlihan *et al.*, 2004); and (iv) the effect of *S. bovis* HC5 on JB1 was bacteriostatic rather than bactericidal.

Bovicin HC5 was bactericidal against *Listeria monocytogenes* at pH 6.5 and 5.5, but only if incubation time was greater than 2 h (Mantovani & Russell, 2003b). Similar effects have been noted for nisin and bacteriocins of *Carnobacterium piscicola* and *Carnobacterium divergens* (Duffes *et al.*, 1999). The trend that favored bacteriocin-producing *S. bovis* isolates occurred before the cells had reached stationary phase and cell-free activity could not be detected (Mantovani & Russell, 2003a). However, recent work indicated that *S. bovis* HC5 had 10-fold more cell-associated than cell-free activity (Houlihan *et al.*, 2004). These results support the idea that cell-associated bovicin HC5 could be more important than cell-free activity.

Bacterial growth in the rumen is driven by ammonia as well as amino nitrogen (Kreth *et al.*, 2005), and *S. bovis* can utilize either of these nitrogen sources (Hungate, 1975). The growth rate advantage of *S. bovis* JB1 over HC5 was nearly eliminated if ammonia was the nitrogen source, but *S. bovis* HC5 lagged for a longer time than JB1. The lag time effect favored bacteriocin-negative isolates, but this trend could be circumvented by increasing the glucose concentration. When the glucose concentration was 7 rather than 2 mg mL^{-1}, the cultures had a longer time to grow, the effect of lag time was less, and bacteriocin-producing bacteria once again dominated the cocultures.

Recent work demonstrated that *C. sticklandii* SR could bind cell-free bovicin HC5, and decrease the amount of bovicin HC5 that could bind *S. bovis* JB1 (Houlihan *et al.*, 2004). Our current work indicates that *C. sticklandii* SR, another bovicin-sensitive bacterium, could shift the *S. bovis* competition from bacteriocin-producing isolates to ones that did not produce bacteriocin. This result indicated that the capacity of bacteriocin-producing bacteria to inhibit a particular target bacterium could be compromised by the presence of other sensitive bacteria. Because total *S. bovis* numbers did not decline, the effect of *C. sticklandii* SR could not be explained by a general inhibition.

Bacteriocin structural genes are often located on plasmids or transposons, but a variety of other genes are also needed for secretion, self-protection, regulation and posttranslational modification (Wolin *et al.*, 1959). *Streptococcus bovis* strains can be differentiated by BOX PCR (Jarvis *et al.*, 2001), and all of the bacteriocin-producing isolates were identical to *S. bovis* HC5. Given this observation, it is very unlikely that bovicin HC5 genes were simply being transferred from *S. bovis* HC5 to *S. bovis* JB1.

It has been argued that bacteriocin-producing bacteria are favored in nutrient-rich environments (Frank, 1994). Immediately after feeding, the rumen resembles a nutrient-rich batch culture (Hungate, 1975), and bacteriocin-producing isolates dominated our batch cultures. However,

in vivo, soluble energy sources are soon depleted, and bacteria must depend on extracellular enzymes that degrade the insoluble substrates. Because the animal often eats many times a day, the rumen can also be compared to an energy-limited continuous culture device. Energy-limited continuous culture devices select for bacteria with the highest affinity (K_s) for the energy source relative to its maximum growth rate (μ_{max}) (Harder & Kuenen, 1977), and our work indicated that this type of selection (μ_{max}/K_s) was a stronger force than bacteriocin production. These observations support the idea that nutrient-rich environments favor bacteriocin-producing strains (Frank, 1994).

Disclaimer

Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable.

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